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Review

Natural antisense (rTSα) RNA induces site-specific cleavage of thymidylate synthase mRNA

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Abstract

The 3′ untranslated region (UTR) of rTSα RNA is complementary (i.e., antisense) to human thymidylate synthase (TS) RNA. When HEp2 cells (human epidermoid carcinoma) progressed from late-log to plateau phase growth, ribonuclease protection assay (RPA) revealed an inverse correlation between the levels of rTSα RNA and TS mRNA, suggesting a possible effect of rTSα RNA on TS mRNA levels. HEp2 cells expressing a Tet-On transactivator were transiently co-transfected with pHook-1 and a construct containing rTSα (protein and antisense RNA), rTSαΔ3′ (rTSα protein only), rTSα-3′ (antisense RNA–luciferase) or luciferase. Transfected cells were selected and evaluated for the effects of induced transgene expression on TS mRNA. Induced expression of transfected rTSα or rTSα-3′, but not rTSαΔ3′ or luciferase, resulted in decreased TS mRNA levels as measured by RPA. These results demonstrated that the antisense region of rTSα RNA is necessary and sufficient for this down-regulation of TS mRNA. RPA for TS mRNA also showed the enhanced appearance of two partial-length protected fragments in rTSα or rTSα-3′ transfected cells. RPA stringency evaluations and primer extension assays indicated that TS mRNA is cleaved in vivo in a site-specific manner. These data demonstrate that rTS gene expression likely plays a role in down-regulating TS through a natural RNA-based antisense mechanism.

Keywords: Chemotherapy; Thymidylate synthase; RNA editing; Antisense RNA

1. Introduction

Thymidylate synthase (TS, EC 2.1.1.45) is the enzyme that catalyzes the methylation of deoxyuridylate to deoxythymidylate (dTMP). This enzymatic reaction provides the sole de novo intracellular source of dTMP, an essential nucleotide precursor for DNA replication and repair. As a result, TS has been a major target in cancer chemotherapy for over 30 years [1]. Since some tumor cell lines resistant to TS inhibitors overproduce the enzyme [2,3], a better understanding of mechanisms underlying the regulation of TS levels is important for the development of cancer therapies involving TS inhibition.

The TS gene is expressed at a much higher level in rapidly proliferating cells than in nondividing cells [4–10]. However, nuclear run-on assays showed that the rate of the TS gene transcription is relatively invariant as cells are activated from a resting to a cycling state [5,11]. Therefore, TS gene expression appears to be controlled mainly at the posttranscriptional level in growth-stimulated cells [12]. Since growing cells are comprised of a large percentage of cells in S phase, which generally parallels changes in TS levels, TS appears to be an S-phase enzyme. This is supported by the evidence that elevated expression of E2F results in increased TS protein levels [13]. However, studies on exponentially growing asynchronous cells have indicated that the levels of TS mRNA [4], TS protein and TS activity [6,10] fluctuate only within a narrow range throughout the cell cycle, with the largest increase occurring during the G_0/G_1 transition [6,10].

In 1993, our laboratory reported the discovery of a naturally occurring antisense RNA to TS (now referred as rTSα EMBL accession #X67098) [14]. The 3′ untranslated region (UTR) of rTSα mRNA is complementary (antisense) to both TS pre-mRNA and TS mRNA (Fig. 1). Natural antisense RNA was first discovered in prokaryotes and has been demonstrated to exert negative control of

Abbreviations: BSA, bovine serum albumin; Cyc, cyclophilin; Dox, doxycycline; RPA, ribonuclease protection assay; TS, thymidylate synthase; UTR, untranslated region

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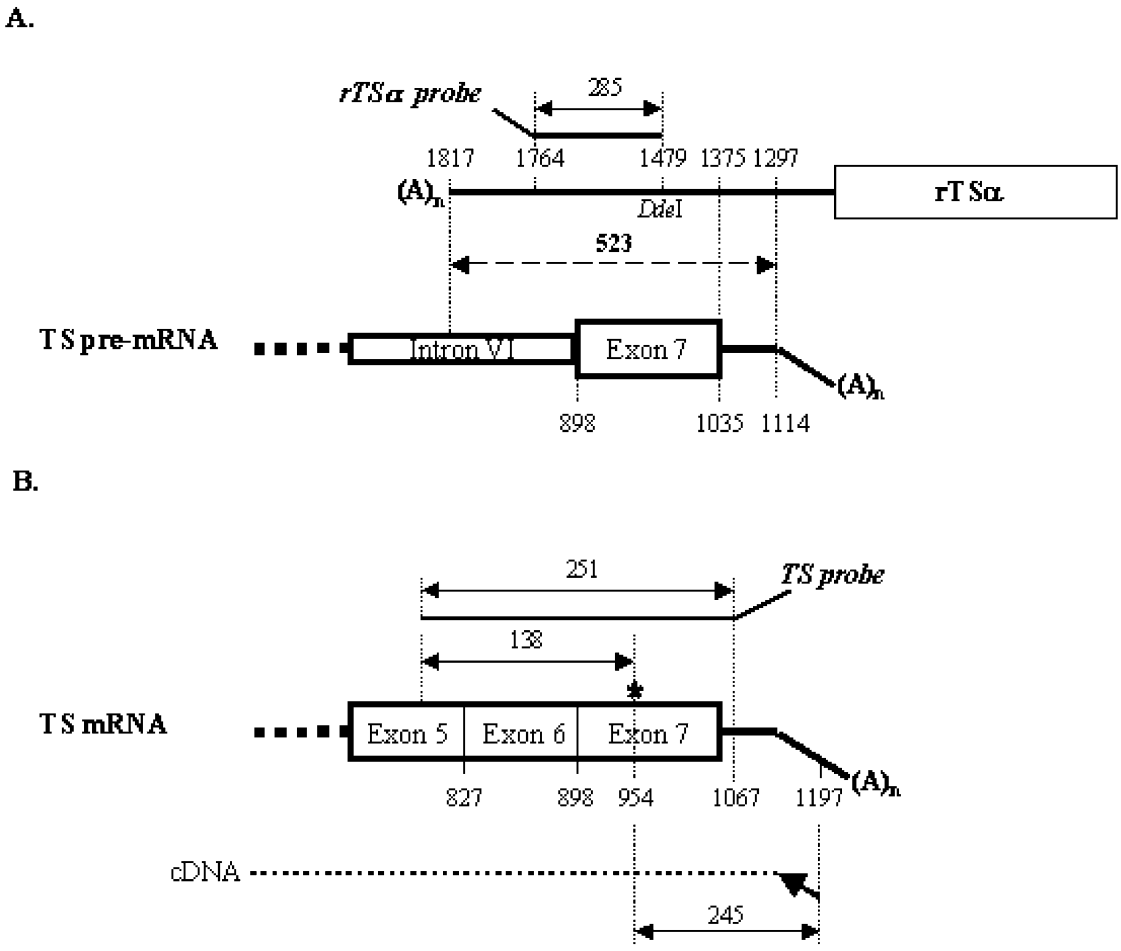

Fig. 1. (A) The relationship between the rTSα mRNA and the TS gene/pre-mRNA. The protein coding region and 3′ UTR of rTSα are indicated by an open box and a solid line, respectively. The two boxes in the TS pre-mRNA represent a portion of intron VI and the protein coding region of exon 7 as indicated. The 3′ UTR of exon 7 is indicated by a solid line. The rest of TS pre-mRNA is represented by a dotted line. Numbers represent nucleotide distance either from the 5′ end of the rTSα RNA or over the spans indicated by double arrows. rTSα mRNA is complementary to TS pre-mRNA over a span of 523 nt, including the first A of the rTSα poly (A) tail. The targeted region (285 nt) of the rTSα riboprobe is also indicated at the top. (B) Targeted region of the TS riboprobe and the expected cDNA products from primer extension assay. Open boxes represent exons 5, 6 and 7 of TS mRNA with the solid line representing the 3′ UTR. The rest of the TS mRNA is represented by a dotted line. The TS probe is represented by a solid line at the top. The full-length protected fragment would be 251 nt as indicated at the top. The primer used in primer extension assays is represented by the left-facing arrow. A TS RNA editing site that generates a mismatch in the TS probe:mRNA duplex is indicated by an asterisk (see Results and Discussion). The protected fragment in RPA resulting from cleavage at the editing site is 138 nt. The predicted length of cDNA produced by primer extension from the TS mRNA cleaved at the editing site is 245 nt.

gene expression at multiple levels, such as transcriptional attenuation, interference with RNA processing, RNA destabilization and translational inhibition [15]. Increasing numbers of naturally occurring antisense RNAs have been identified in eukaryotes, raising the possibility that they might play a role in the regulation of eukaryotic gene expression [16,17]. However, in mammalian cells, the functions of natural antisense RNAs in regulating the expression of their counterpart (sense) genes have not been established.

In the present study, we show that rTSα RNA and TS mRNA levels vary inversely when cell growth transits from late-log to plateau phase. We demonstrate that the antisense portion of rTSα mRNA alone can down-regulate TS mRNA levels in transiently transfected cells. Our data also show that this down-regulation is associated with increased site-specific cleavage of TS mRNA.

2. Experimental procedures

2.1. Plasmids and probes

The plasmids for the Tet-On gene expression system (pUHD 172-1 neo, pUHD 10-3 and pUHC13-3) were obtained from Dr. Hermann Bujard [18]. To construct the expression vector for rTSα, we cloned the *Eco*RI–*Hin*dIII fragment of rTSα containing the full-length cDNA into the *Eco*RI and *Xba*I sites of pUHD10-3. The *Hin*dIII site of the rTSα insert and *Xba*I site of pUHD10-3 were partially filled

in by Klenow enzyme (Life Technologies) to generate cohesive ends. The expression vector for rTSα protein without antisense RNA (rTSαΔ3′) was constructed by cloning the *Eco*RI–*Blp*I (blunt-ended) fragment of the rTSα cDNA into the *Eco*RI and *Xba*I sites (blunt-ended) of pUHD 10-3. The 3′ UTR was generated by PCR using primers containing the *Sty*I recognition sequence (boldface type) (5′-primer: CGTGACC**CCAAGG**AAGTGCTCAGC-CCCA-ACAACT, 3′-primer: GCGTGCT**CCTTGG**TGAC-TGTGTTCCTGTCTTTAA). The vector for expressing rTSα antisense RNA without rTSα protein (rTSα-3′) was constructed by cloning the PCR generated fragment into the *Sty*I site of pUHC13-3. The sequences of all cloned cDNAs were confirmed by sequencing at the RPCI Biopolymer Facility.

The template for in vitro transcription used to generate the TS riboprobe was *Sma*I linearized plasmid pTS-PCRmut1 [19]. The targeted region of this TS probe includes part of exon 5 (10 nt), exon 6 (72 nt) and part of exon 7 (169 nt) (Fig. 1B). The template for the rTSα riboprobe was *Dde*I linearized plasmid pTOPO/G4, produced by inserting a PCR fragment from H630 cell genomic DNA corresponding to rTSα mRNA nt 1326–1764 (Fig. 1A) into pCR2.1-TOPO (Invitrogen). The template for making the cyclophilin (Cyc) riboprobe was pTRI–Cyc–Human (Ambion). The lengths of TS, rTSα and Cyc probes (and protected fragments) were 265 (251), 354 (285) and 165 (103) nt, respectively. All ^{32}P-RNA probes were produced by in vitro transcription using Maxi-Script/T7 polymerase kits (Ambion) as recommended by the manufacturer using [α-^{32}P]CTP (800 Ci/mmol, 10 mCi/ml, NEN Life Science Products). DNA templates were removed by DNaseI digestion. All ^{32}P-labelled riboprobes were purified by 5% polyacrylamide/8 M urea gel electrophoresis. Specific radioactivities of the TS, rTSα and Cyc probes used were 1.8×10^9, 1.5×10^9 and 8×10^7 cpm/μg, respectively.

2.2. Cell culture and transfections

The HEp2 cell line (human epidermoid carcinoma, ATCC CCL 23) was maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Life Technologies) at 37 °C in a 5% CO_2 humidified atmosphere. The HEp2 cell line was chosen for study because of its low level of endogenous rTSα expression and because of a robust response to doxycycline (Dox) induction in preliminary co-transient transfection assays using pUHD172-1 neo and pUHC 13 3 (data not shown).

For the cell growth experiment (Fig. 2A), HEp2 cells were seeded on multiple plates (8.0×10^5 cells/10 cm plate) and allowed to grow for different periods of time. Cells were extracted to obtain both RNA and protein using TRIZOL reagent (Life Technologies) as recommended by the manufacturer. For each time point, the cell number in a duplicate plate was determined using a Coulter counter.

For transfections to establish the Tet-On gene expression system, pUHD 172-1 neo was first stably transfected into HEp2 cells using lipofectin (Life Technologies) as recommended by the manufacturer. Clones were selected in the presence of G418 (500 μg/ml) and were screened for their ability to be induced for luciferase expression by transient-transfection assays. Clone P9E was Dox-inducible for luciferase expression (100-fold), and was used in subsequent experiments. For each co-transient transfection with pHook-1 (Invitrogen) [20] and one of the four constructs (rTSα, rTSαΔ3′ rTSα-3′ and luciferase, Fig. 3A), cells at about 50% confluence were harvested and washed with ice-cold PBS. The cell pellet was resuspended in ice-cold PBS at a density of $\sim 5.7\times10^7$ cells/ml, and 2.1 ml of cell suspension was mixed with 150 μg plasmid DNA (molar ratio of pHook1/experimental construct = 10:1) and incubated on ice for 10 min. The cells plus DNA mixture was evenly transferred into three prechilled cuvettes (BTX, 4 mm gap). Electroporation was performed using a BTX ECM 600 electroporator (400 V, 800 μF, 48 Ω). After the pulse, the cuvettes were returned to ice for 10 min. The electroporated cells were pooled and resuspended with RPMI 1640 medium containing 10% fetal bovine serum and 300 μg/ml G418, and plated on 25 15-cm plates. After 20 h, transfected cells were selected with Capture-Tec™ Beads (200 μl bead slurry, Invitrogen) as recommended by the manufacturer. The selected cells (~ 20% of total viable cells) were plated at $\sim 1.0\times10^6$ cells/10 cm plate and returned to culture in the presence or absence of Dox (1.0 μg/ml).

2.3. Western blot

Protein concentrations were determined using a BCA™ Protein Assay Kit with bovine serum albumin (BSA) as a standard as described by the manufacturer (Pierce). Proteins (35 μg) were resolved by 10% SDS polyacrylamide gel electrophoresis and immunoblotting was performed as previously described [21].

2.4. RNase protection assay (RPA)

RPA to measure TS mRNA or rTSα RNA was carried out using the HybSpeed RPA kit (Ambion) essentially as described by the manufacturer. The amount of each probe needed to achieve a molar excess over its target RNA was determined by pilot experiments that established the linearity of the assay. Cyc mRNA was also measured to serve as an internal standard for quantitating protected fragments [22]. Briefly, 5 μg of RNA, 6×10^4 cpm of TS or rTSα riboprobe and 3×10^4 cpm of Cyc riboprobe were mixed and co-ethanol-precipitated with yeast RNA (45 μg) as carrier. The RNA pellet was dissolved in 10 μl of hybridization buffer by heating at 95 °C with vortexing. Hybridization was performed at 68 °C for 18 h, and RNase digestions were carried out at 37 °C for 40 min with RNase A/T1 (1:100),

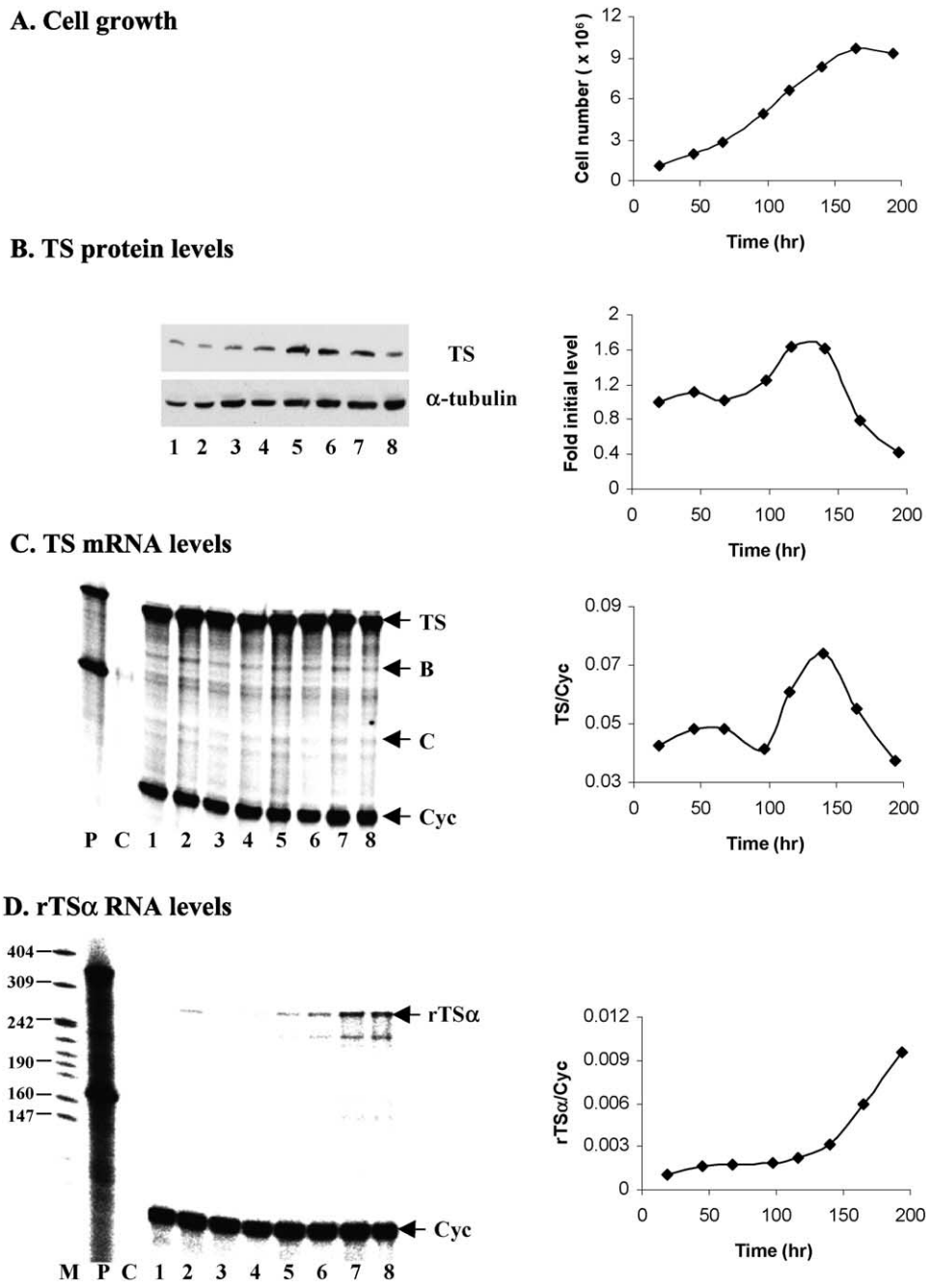

Fig. 2. Variation of TS protein, TS mRNA and rTSα RNA levels during asynchronous cell growth. (A) HEp2 cell growth. Cells were seeded at time 0 h (8×10^5 cells/10 cm plate) and extracted at 19, 45, 67, 97, 116, 140, 166 and 194 h after seeding as described in Experimental procedures. The doubling time of this cell line is 40 h. (B) Lanes 1–8: Proteins obtained from different time points of cell growth (19–194 h) were subjected to Western blot analysis for TS and α-tubulin as described in Experimental procedures (left). The change of TS protein levels with cell growth normalized to α-tubulin is shown on the right. Panels (C) and (D) are the results of RPA for TS mRNA and rTSα RNA, respectively. For all RPA experiments, Cyc mRNA was used as an internal standard. Left panels show the phosphorimages of electrophoretic gels and positions of relevant protected fragments are indicated to the right of the images (TS, 251 nt TS fragment; B, 195 nt TS fragment; C, 138 nt TS fragment; Cyc, 103 nt Cyc fragment; rTSα 285 nt rTSα fragment). Lanes 1–8: Cellular RNAs from different time points of cell growth (19–194 h) were hybridized with TS and Cyc probes. M: pBR322/*Msp*I molecular weight marker. Lanes P and C: The TS or rTSα and Cyc probes were hybridized with yeast RNA either with (C) or without (P) RNase digestion. In lane P, discrete bands can be seen at the predicted lengths of 265 (TS), 354 (rTSα) and 165 nt (Cyc), whereas lane C shows complete digestion of unprotected probes. The graphs on the right show the molar ratios of TS mRNA (B) or rTSα RNA (D) to Cyc mRNA at each time point of cell growth (calculated according to the formula given in Experimental procedures).

unless otherwise indicated. Protected fragments were resolved by 5% polyacrylamide/8 M urea gel electrophoresis and quantitated by phosphor screen imaging (Molecular Dynamics Storm 860 with a setting of 750 V, 200 μm). The lower specific activity of the Cyc probe allowed simultaneous quantitation of TS mRNA and rTSα RNA.

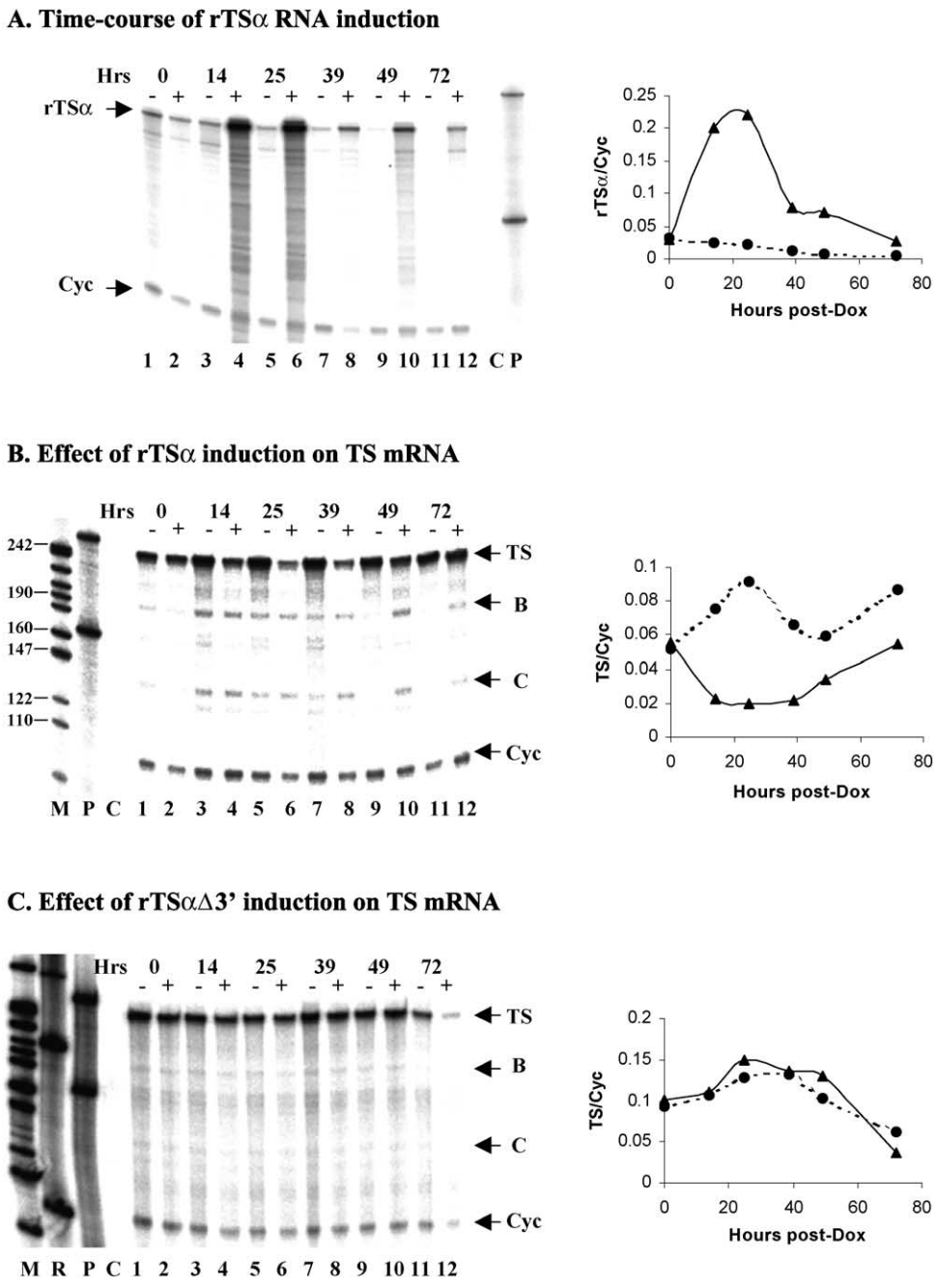

Fig. 3. RPA results for rTSα and rTSαΔ3′ transiently transfected cells. (A) Time course analysis of rTSα RNA induction in rTSα transfected cells. (B,C) Time-course analyses of TS mRNA levels in rTSα and rTSαΔ3′ transfected cells, respectively. Panels are labeled as in the legend to Fig. 2. Lanes 1–12: RNA samples obtained at 0–72 h after adding Dox (0 and 1.0 μg/ml as indicated by " – " and " + ", respectively) were subjected to RPA as described in Experimental procedures. R:RNA marker (Ambion's century marker). For each graph on the right, the solid line with triangles represents induced cells (+ Dox) and the dotted line with circles uninduced cells (– Dox).

2.5. Primer extension assay

Cellular RNA (20 μg) was mixed with 0.1 μmol primer (ACATTTGCCAGT-GGCAACAT, Fig. 1) and incubated at 70 °C for 5 min. The reverse transcription reaction (60 μl) contained sample RNA, the primer, 10 mM DTT, 500 μM each of dATP, dGTP, dTTP, 20 μM dCTP, 17 μM [α-^{32}P]dCTP (3000 Ci/mmol, 10 mCi/ml, NEN Life Science Products), 1 × first strand buffer (Life Technologies) and 400 units of Superscript II reverse transcriptase (Life Technologies). The reaction was carried out by incubation at room temperature for 15 min followed by heating at 37 °C for 1 h. The reaction was terminated and RNA hydrolyzed by adding EDTA (40 mM) and NaOH (400 mM) and heating at 68 °C for 30 min. The DNA products were ethanol-precipitated, washed with 70% ethanol, and dis-

solved in water. An excess amount of biotinylated TS transcript generated from the *Hin*dIII linearized pTOPO/G4 by T7 polymerase was added and the nucleic acid mixture ethanol-precipitated. The pellet was dissolved in 10 μl of hybridization buffer (Ambion's HybSpeed RPA kit) by heating at 95 °C for 5 min with vortexing. Hybridization was performed by incubation at 56 °C for 18 h. Streptavidin superparamagnetic beads suspension (40 μl, Sigma) was washed twice with 1.0 ml of TNE 300 (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.1% SDS and 300 mM NaCl) and resuspended in 100 μl TNE 300. Forty micrograms of yeast RNA was added to the suspension and incubated at 37 °C for 30 min with mixing by rotation. The pretreated streptavidin beads in 100 μl of TNE 300 were added to the hybridization reaction followed by incubation at 37 °C for 30 min with mixing by rotation. One milliliter of TNE 300 was then added and mixed well by vortexing. The supernatant was removed after placing the tube in a magnetic stand. This washing procedure was repeated five times at which time the ^{32}P-cpm in the supernatant decreased to

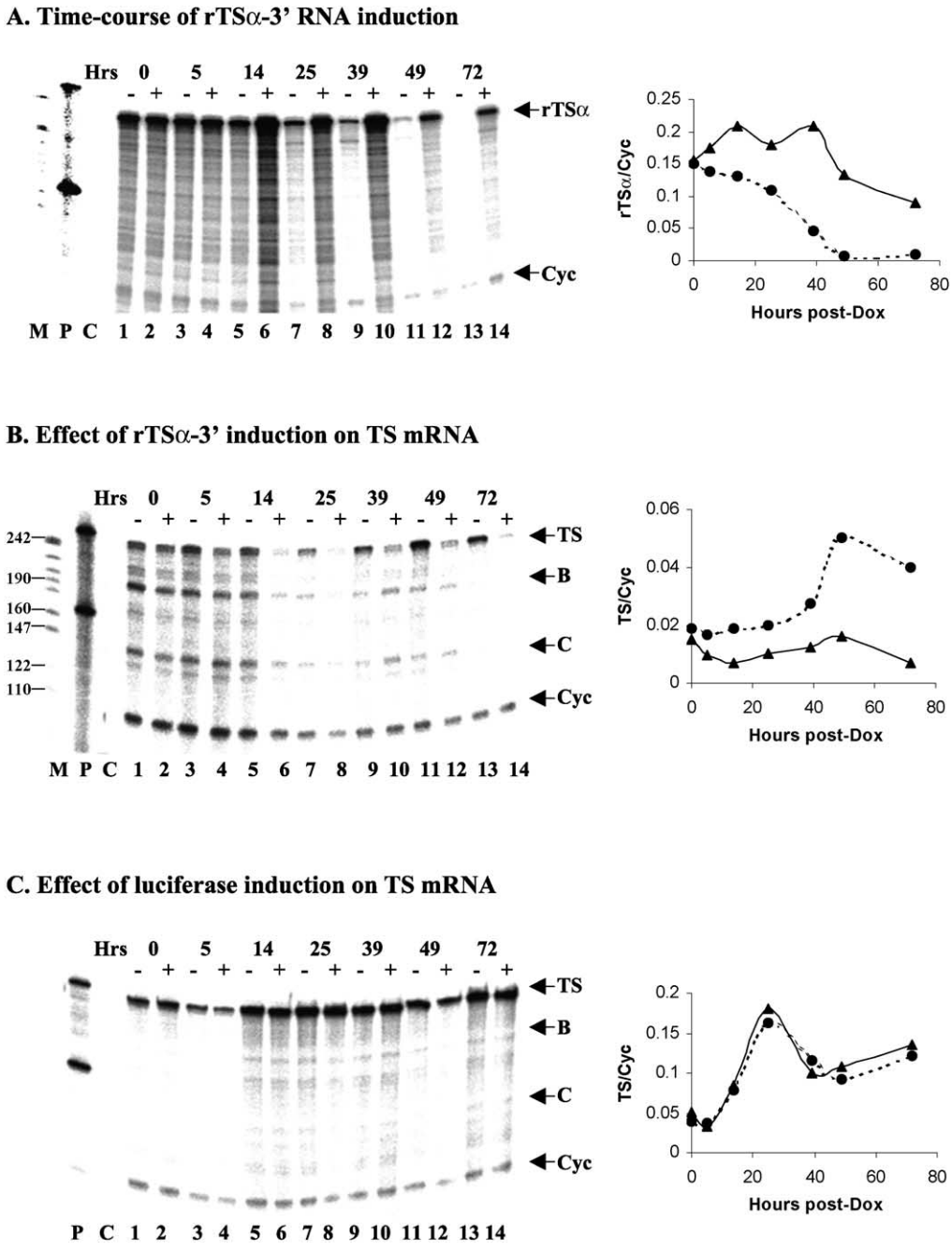

Fig. 4. Results for rTSα-3′ and luciferase transiently transfected cells. (A) Time course analysis of rTSα RNA induction in rTSα-3′ transfected cells. (B,C) Time-course analyses of TS mRNA levels in rTSα-3′ and luciferase transfected cells, respectively. Panels are labeled as in the legends to Figs. 2 and 3.

background level. The bound ^{32}P-cDNAs were then recovered by heating the beads in 40 μl of gel loading buffer (95% formamide, 18 mM EDTA, 0.025% SDS, xylene cyanol and bromophenol blue) at 100 °C for 5 min and resolved by 5% polyacrylamide/8 M urea gel electrophoresis.

3. Results

3.1. TS mRNA levels decrease while rTSα RNA levels increase as cell growth progresses from late-log to plateau phase

It has previously been demonstrated that TS activity and protein levels do not change much during the log-phase of asynchronous cultured cell growth but decrease when cells reach plateau phase growth [6,10]. To investigate whether changes in TS mRNA levels behave this way in HEp2 cells, and how rTSα RNA levels vary in relation to TS mRNA, the levels of both TS mRNA and rTSα RNA during asynchronous cell growth were determined by RPA. HEp2 cells were seeded and extracted at the indicated time points to measure the levels of TS and rTS gene expression (Fig. 2). As shown in Fig. 2A, cell growth began to reach plateau phase at 166 h after seeding. Consistent with previous findings [6,10], the levels of TS protein fluctuated within a narrow range (1.6-fold) during log-phase growth (45 to 116 h, Fig. 2B) but decreased 4-fold when cells transited from late-log to plateau phase growth (140 to 194 h, Fig. 2B). In agreement with the decline in TS protein during the transition to plateau phase, TS mRNA levels decreased 2-fold (140 to 194 h, Fig. 2C). In contrast to TS mRNA, rTSα RNA levels increased more than 3-fold when cells progressed from late-log to plateau phase (140 to 194 h, Fig. 2D). The inverse variation between the levels of TS mRNA and rTSα RNA during the late-log and plateau stages of cell growth (140 to 194 h, Fig. 2C,D) suggested a possible role of rTSα RNA in the down-regulation of TS mRNA levels. RPA for TS mRNA also showed the appearance of other fragments smaller than the full-length protected probe (Fig. 2C). Two of these fragments (B, 195 nt and C, 138 nt) were later found to correspond to in vivo TS mRNA cleavage products.

3.2. TS mRNA levels can be down-regulated by rTSα antisense RNA

To investigate the possibility that rTSα RNA can mediate a decline in TS mRNA levels, cell transfections were performed. Since we were unable to obtain a permanent stably transfected cell line expressing rTSα [23], rTSα transient co-transfections of HEp2 cells containing the Tet-On transactivator [18] with pHook-1 and rTSα or rTSαΔ3′ were carried out. Co-transfection with pHook-1 allows specific selection of transfected cells for evaluation [20]. After selection, cells were seeded in the presence or absence of Dox and extracted at different time intervals to obtain both RNA and protein. RPA clearly showed that increased expression of rTSα RNA led to decreased TS mRNA levels

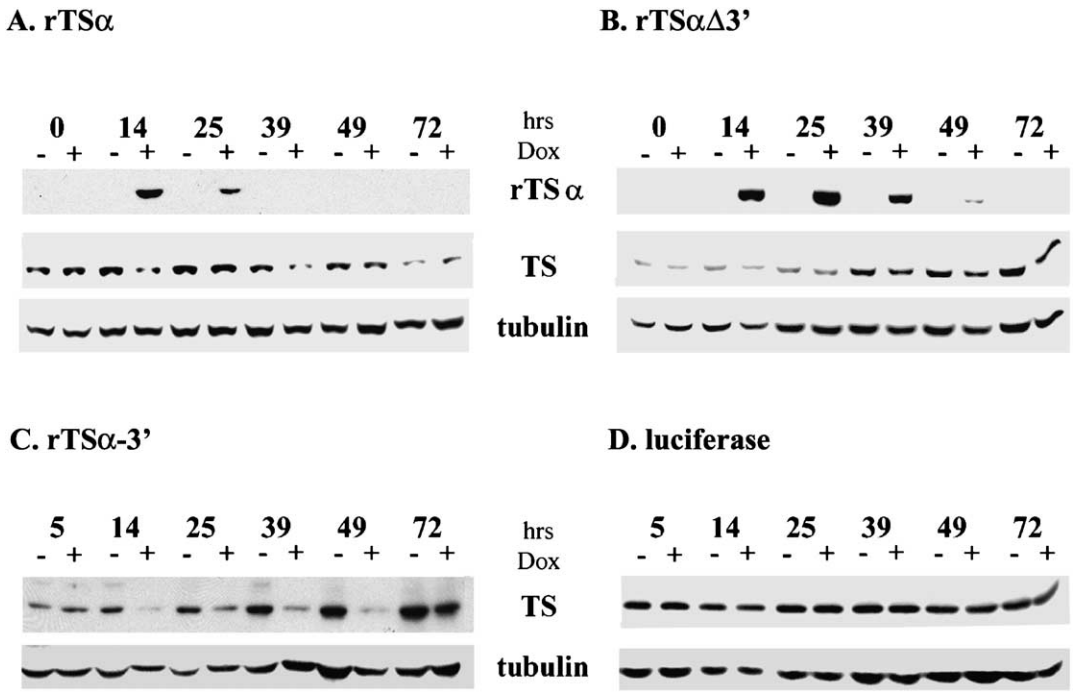

Fig. 5. Western blots showing rTSα and TS protein expression after transient transfections with rTSα (A), rTSαΔ3′ (B), rTSα-3′ (C) and luciferase (D). Each blot was sequentially probed for rTSα, TS and α-tubulin as described in Experimental procedures. rTSα was not visible in the blots shown in panels C and D.

(Fig. 3A,B). At 25 h post-Dox, the TS mRNA level decreased by 80%. TS protein levels were also down regulated by the increased expression of rTSα (Fig. 5A). After 39 h post-Dox, rTSα RNA was still inducible with Dox (Fig. 3A) but rTSα protein was no longer detected (Fig. 5A). This suggested that the down-regulation of TS mRNA levels by the induced expression of rTSα was most likely mediated by rTSα RNA rather than by rTSα protein. This conclusion is supported by data obtained from transfections performed with rTSαΔ3′, which contains the rTSα protein coding region but does not have a region of RNA antisense to TS RNA (Figs. 3C and 5B). Higher levels of rTSα protein have been induced for a longer duration in comparison with the rTSα mRNA transfected cells (Fig. 5B). The effects of rTSα protein (rTSαΔ3′) induction differed from those of rTSα mRNA. The rTSαΔ3′ transfected cells did not display significant changes in TS mRNA (Fig. 3C) but did show alterations in TS protein levels after 39 h (Fig. 5, compare B with C).

Down-regulation of TS by rTSα mRNA but not by rTSα protein suggested that the 3′-antisense portion of rTSα RNA might be sufficient for the observed negative regulation of TS mRNA. To investigate this further, transient transfections with rTSα-3′ or luciferase were performed. While increased expression of luciferase had no effect on the levels of either TS mRNA (Fig. 4C) or protein (Fig. 5D), induced expression of rTSα-3′ resulted in decreased levels of both TS mRNA (Fig. 4A,B) and protein (Fig. 5C).

3.3. rTSα RNA induces site-specific cleavage of TS mRNA at physiologic cleavage sites

As shown in Fig. 2C, RPA of TS mRNA in non-transfected HEp2 cells showed numerous smaller protected fragments in addition to the full-length protected fragment (251 nt). Interestingly, the intensity of two of these fragments (B, 195 nt and C, 138 nt) were increased in rTSα antisense RNA transfected cells in comparison with non-transfected cells and rTSαΔ3′ or luciferase transfected cells (Figs. 3B and 4B compared with Figs. 2C, 3C and 4C). The levels of the 195 (B) and 138 nt (C) bands were found to correlate with the levels of rTSα antisense RNA in rTSα antisense RNA transfected cells. When rTSα or rTSα-3′ transfected cells were induced to express higher levels of the antisense RNA (Figs. 3A and 4A), the levels of these two smaller fragments relative to TS mRNA levels also increased(Fig. 6). With the decline of rTSα antisense RNA in uninduced cells during 72 h after seeding rTSα or rTSα-3′ transfectants (Figs. 3A and 4A), the intensity of these two fragments also declined (Fig. 6). During the first 39 h, rTSα-3′ transfected cells displayed higher, uninduced levels of the antisense RNA compared with rTSα transfected cells (Fig. 6, compare A and B). Correspondingly, rTSα-3′ transfected cells in the absence of Dox had an increased relative abundance of these two bands (Fig. 6, compare A and B).

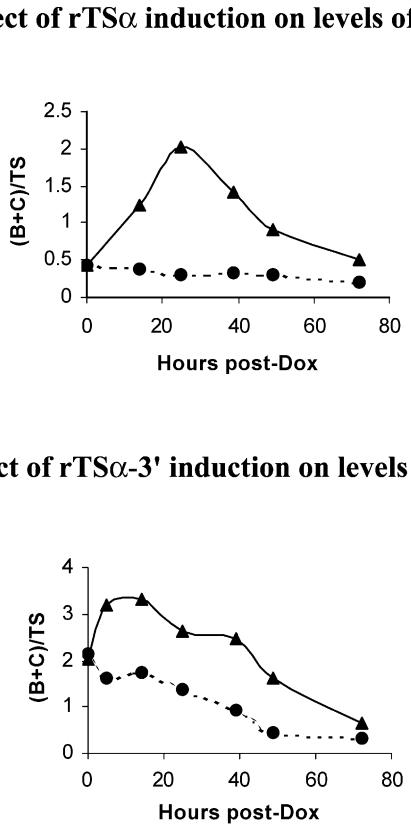

Fig. 6. Levels of two protected fragments (i.e., B and C) relative to TS mRNA from the time-course study of rTSα (A) and rTSα-3′ (B) transfected cells. The values in panels A and B are based on quantitations of the relevant protected fragments as shown in the gel images of Figs. 3B and 4B, respectively. The (B + C)/TS molar ratio is calculated as the sum of these two fragments (i.e., (B + C)/Cyc) divided by the amount of TS mRNA (i.e., TS/Cyc).

The origin of the 195 (B) and 138 (C) nt protected fragments could be cleavage of TS probe:mRNA hybrids due to mismatches or transient destabilization (i.e., breathing) during RPA or they could represent in vivo cleaved TS mRNA molecules. Resolving these possibilities is important because the complementarity of rTSα and TS RNAs suggests that rTSα:TS RNA hybrids could form in vivo and serve as substrates for adenosine deaminases that act on RNA (ADAR) [16,24,25]. Our laboratory has identified five editing sites (adenosine to inosine) in TS pre-mRNA in the region of TS RNA complementary to rTSα RNA (unpublished data). One editing site is predicted to generate a mismatch with the ^{32}P-labeled probe that would produce a 138 (C) nt protected fragment in the RPA (Fig. 1). The abundance of this protected fragment along with the 195 (B) nt fragment increased in response to elevated levels of rTSα antisense RNA in rTSα and rTSα-3′ transfected cells (Fig. 7), suggesting that the 138 (C) nt fragment could arise by cleavage at an RNA editing site.

To verify that these two species (B and C) are not cleavage products resulting from RNA duplex breathing during RPA, RNase digestions were performed at different

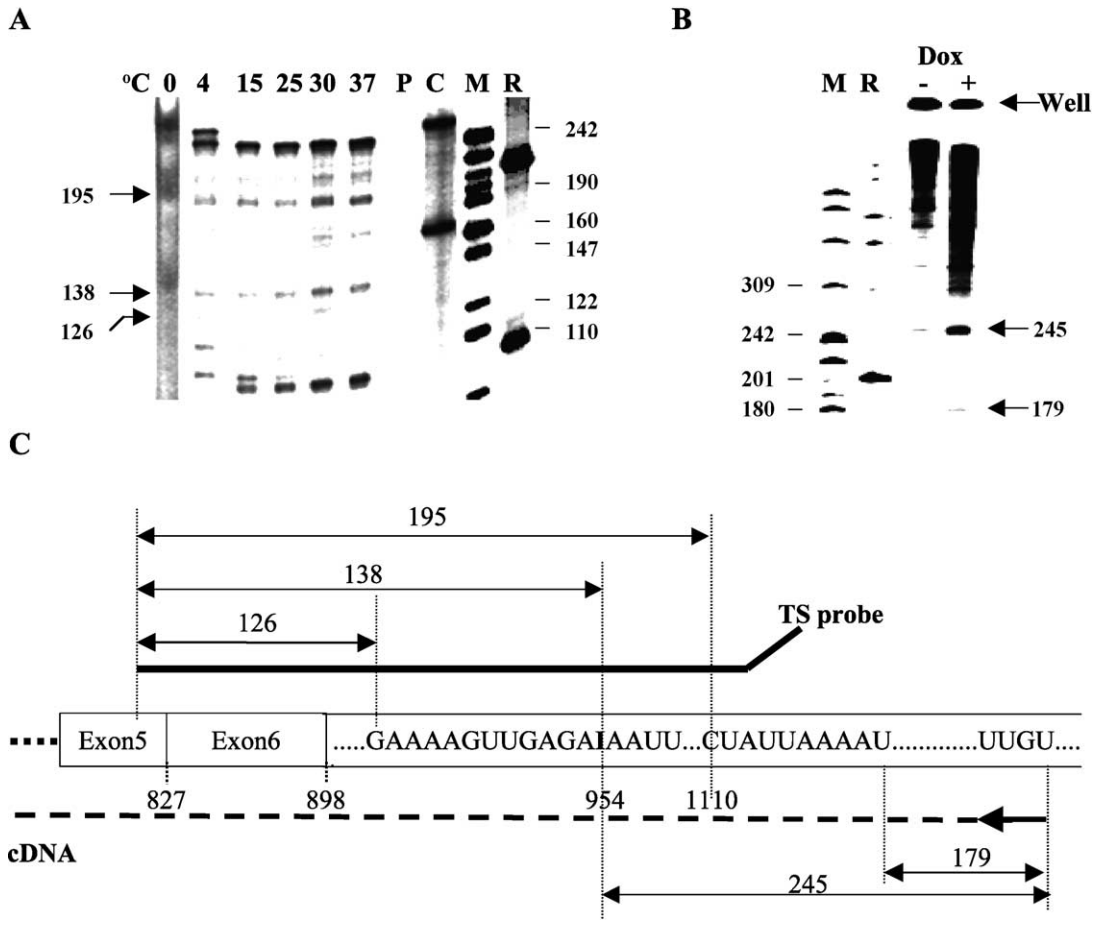

Fig. 7. (A) Results of RPA for TS mRNA performed with varying stringency of RNase digestion. Cellular RNA from rTSα-3′ transfected cells was subjected to RPA as described in Experimental procedures except that the RNase digestion step was performed over a range of temperatures (0–37 °C) as indicated. (B) Results of primer extension assays. Assays were performed as described in Experimental procedures to detect in vivo generated TS mRNA fragments from rTSα-3′transfected cells (with or without Dox). (C) Schema showing the protected fragments (195 and 138 nt) of RPA and their corresponding ^{32}P-cDNA products (179 and 245 nt) produced by the primer extension.

stringencies by altering the temperature (0 to 37 °C). As shown in Fig. 7A, the amounts of these two fragments relative to TS mRNA remained unchanged over a wide range of temperatures. The levels were relatively unchanged even at 4 °C where the unprotected 3′-overhang of the TS probe:TS mRNA duplex was not digested, leaving the entire probe intact. This result suggests that the protected fragments detected by our RPA are less likely to have been produced in vitro by RNase digestions of unstable duplexed regions of the TS probe:mRNA hybrids.

The likelihood that TS mRNAs are cleaved in vivo in response to increased levels of rTSα antisense RNA was investigated further using a primer extension assay. Cellular RNA extracted from rTSα-3′ transfected cells (39 h post-Dox) were reverse transcribed using a TS gene specific primer (Fig. 1) in the presence of [α-^{32}P]dCTP. The ^{32}P-cDNA products are expected to have lengths reflecting TS in vivo cleavage sites. If TS mRNA is cleaved in vivo, a limited number of discrete ^{32}P-cDNA fragments should be detectable provided that the cleaved RNA molecules are relatively stable during the time course of study. To minimize the background generated from nonspecific priming, TS ^{32}P-cDNAs were selected by hybridization to biotinylated TS RNA prepared by in vitro transcription. Hybridized TS ^{32}P-cDNAs were then purified by affinity chromatography with immobilized streptavidin. As shown in Fig. 7B, increased amounts of TS ^{32}P-cDNAs of 245 and 179 nt are observed for primer extension performed with RNA from transfected cells induced to express rTSα antisense RNA. The size of the ^{32}P-cDNA (245 nt) is that for a cDNA produced from TS mRNA cleaved at one of the two editing sites identified in exon 7 of TS pre-mRNA (unpublished data) (Fig. 7C). This ^{32}P-cDNA corresponds to the 138 (C) nt RPA protected fragment (B and C in Fig. 4B). Therefore, TS mRNA was cleaved in vivo at an editing site in response to rTSα antisense RNA. The 179 nt ^{32}P-cDNA was the result of extension to an adenosine site in an AU rich region of TS mRNA as shown in Fig. 7C. This ^{32}P-cDNA corresponds to an in vivo cleaved TS mRNA molecule with a cleavage site 9 nt downstream from the site that would

generate the 195 nt protected fragment (B) observed in the RPA. This difference probably reflects the AU-rich nature of the intervening region between these two sites (Fig. 7C) that likely gets degraded during the RPA (see Discussion).

4. Discussion

The rTS gene was originally identified by sequencing a cDNA clone for a naturally occurring antisense RNA to human TS mRNA [14]. These two genes are located at the same locus of chromosome 18 [26]. The rTS gene overlaps the 3′ end of the TS gene [27], and is transcribed convergently with TS. Through alternative splicing, the rTS gene transcript produces two mRNAs, rTSα and rTSβ [28,29]. The 3′ UTR of rTSα RNA is complementary to TS RNA near its 3′ end including exon 7 and part of the last intron (Fig. 1). As an initial investigation into the possible role of rTSα RNA in the growth regulation of TS gene expression, we first evaluated rTSα RNA and TS mRNA levels during cell growth to determine their relationship. An inverse relationship was found as cells progressed from late-log to plateau phase (140 to 194 h in Fig. 2: C vs. D). In agreement with the decline of TS mRNA levels, TS protein levels also decreased (Fig. 3B). Although rTSα RNA levels increased as cell growth progressed from late-log to plateau phase, rTSα protein remained undetectable (data not shown). The low-level expression of rTSα protein in HEp2 cells is consistent with our previous observations for other human tumor cell lines [21]. Because of the complementarity between these two RNAs, the association between increased rTSα RNA levels and decreased TS mRNA levels suggests that rTSα RNA might be involved in the down-regulation of TS gene expression.

The possibility that rTSα RNA down-regulates TS mRNA was further investigated through transient transfections. Transient transfection experiments performed with rTSα mRNA, rTSαΔ3′, rTSα-3′ and luciferase as the inducible genes demonstrate that rTSα antisense RNA can down-regulate the levels of TS mRNA. Increased expression of rTSα mRNA or rTSα-3′ but not rTSαΔ3′ or luciferase (Figs. 3 and 4) resulted in decreased TS mRNA levels. These results indicate that the antisense RNA portion of rTSα RNA is both necessary and sufficient for the observed down-regulation of TS mRNA levels.

In addition to the expected protected fragment (251 nt) corresponding to a perfect hybrid of the ^{32}P-probe with TS mRNA, RPA of TS mRNA displayed other smaller fragments. Among these smaller fragments, two discrete bands (B, 195 nt and C, 138 nt) are of particular interest because their intensities increased in the rTSα antisense RNA transfected cells (Figs. 3B and 4B compared with Figs. 2C, 3C and 4C). Further investigations into the nature of these two fragments shed some light on the mechanism for rTSα antisense RNA mediated down-regulation of TS mRNA levels in transiently transfected cells and nontransfected cells as well. There are three possible sources for the appearance of these two bands. First, they might result from the cleavage at some transiently destabilized regions of the TS probe:mRNA duplex during RNase digestion. If this is the case, the levels of these two bands relative to TS mRNA should be identical among all the samples since they were subjected to the same RPA procedure. Since their relative abundance increased in response to the increased levels of rTSα antisense RNA in transfected cells (Fig. 7) and were not affected by altering the stringency of the RNase digestion step of the RPA (Fig. 7A), this possibility can be ruled out. Secondly, some mismatches in the TS probe:mRNA duplex might be susceptible to RNase digestion thus leading to the production of partial-length protected probes. In rTSα antisense RNA transfected cells, the correlation between the increased intensity of these two fragments and the elevated levels of rTSα antisense RNA suggests that the antisense RNA might induce TS mRNA modifications leading to mismatches in the TS probe:mRNA hybrid. This possibility is supported by the finding that TS pre-mRNA is edited (adenosine to inosine) at five sites across the intron 6–exon 7 junction [30]. One editing site in the region targeted by the ^{32}P-labeled TS probe (Fig. 1B) is predicted to generate a mismatch that would produce a 138 nt protected fragment upon cleavage in the RPA (Fig. 1B). Finally, it is also possible that these two fragments might result from in vivo cleaved TS mRNA molecules. This possibility is more likely because the relative intensity of these two bands remained at a similar level even when RNase digestions were performed at temperatures when the mismatched duplexes should be stabilized (Fig. 7A). The detection of elevated levels of a 245 nt ^{32}P-cDNA as a result of primer extension of TS mRNA (Fig. 7B), corresponding to the 138 nt protected fragment in RPA (Fig. 7C), demonstrated that TS mRNA was indeed cleaved in vivo and that cleavage occurred predominantly at this editing site. Another ^{32}P-cDNA of 179 nt corresponds to an adenosine site of an AU-rich region in TS mRNA (Fig. 7B,C). The RNA fragment upstream of this in vivo cleavage site was detected by RPA as the protected 195 nt fragment either because the AU-rich end of the probe:mRNA duplex was susceptible to RNase digestion or because the AU-rich region was degraded in vivo subsequent to cleavage. The 126 nt fragment observed in RPA may be derived from the 138 nt fragment due to RNase-susceptibility of the AU-rich region (AAAAGUUGA) at the end of the duplex formed between the TS probe and mRNA (Fig. 7C). This appears to be likely since the 126 nt fragment diminished in abundance with decreasing temperatures during RNase digestion (Fig. 7A). Consistent with the RPA data, increased expression of rTSα antisense RNA led to increased levels of both ^{32}P-cDNA fragments of 245 and 179 nt, indicating that more TS mRNA molecules were cleaved in vivo in response to the elevated antisense RNA levels.

The 195 nt (B) and 138 nt (C) protected fragments in RPA of TS mRNA were also observed in the nontransfected

HEp2 cells (Fig. 2C). This suggests that the cleavage of TS mRNA induced by rTSα antisense RNA in the transient transfection assays also occurs in the more physiological environment of nontransfected cells. One possible mechanism for the demonstrated down-regulation of TS mRNA levels by rTSα RNA is through duplex formation between their complementary regions. In vivo hybrids have been reported between antisense n-cym RNA and a subset of sense n-myc RNA [31] and in the case of mbp sense and antisense transcripts [32]. The current study provides the evidence for one possible consequence of such duplex formation, site-specific cleavage of TS mRNA. Antisense (PSV-A) RNA-mediated RNA hybrid degradation resulting in down-regulation of the sense RNA has also been implicated in *Dictyostelium* [33]. It was thought that dsRNA-specific RNases might exist in vivo and are responsible for the rapid degradation of duplex RNAs. Indeed, enzymes with this specificity have been described and partially purified [34–36] but their physiological functions remain to be established.

In summary, expression of rTSα RNA down-regulates TS mRNA through an antisense-mediated mechanism. The levels of rTSα antisense RNA and TS mRNA vary inversely when cell growth progresses from late-log to saturation phase (Fig. 2C,D). Additionally, increased expression of the antisense RNA portion of rTSα but not rTSα protein leads to decreased levels of TS mRNA (Figs. 3 and 4). One of the contributors for this decline of TS mRNA is increased site-specific cleavage, particularly at a TS RNA editing site (Fig. 7), which also occurs in nontransfected cells. Therefore, the negative regulation of TS mRNA levels by rTSα RNA is likely mediated by increased TS RNA editing followed by cleavage.

5. Conclusions and perspectives

Based upon the data presented here and the previous observation that most rTSα RNA is nuclear, we suggest that rTSα RNA could play a role in the down-regulation of TS RNA in vivo. While induction of transfected rTSα antisense RNA can cause the down-regulation of TS mRNA, we suspect that the rTSα RNA acts to cause RNA editing with either simultaneous or subsequent cleavage of the TS pre-mRNAs at the editing site(s) in vivo. The implications for chemotherapy will depend upon the mechanism used for the activation of this pathway. For example, if the antisense RNA is constantly in excess to the TS pre-mRNA in the nucleus, then activation of this pathway by some signaling mechanism suggests that this could be exploited in combination with small molecule inhibitors of TS to help prevent new synthesis of TS protein (dependent upon turnover of the TS mRNA). Our laboratory is actively pursuing this possibility.

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